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Lipospheres as a Vaccine Carrier System: Effects of Size, Charge, and Phospholipid Composition

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ABSTRACT

This study describes a new fat-based vehicle (liposphere) that has been developed as a carrier for vaccines. Manufacture of lipospheres is accomplished by gently melting neutral fat in the presence of phospholipid and antigen, and then dispersing the melted mixture in an aqueous suspension by vigorous shaking. Upon cooling of this mixture a phospholipid-stabilized solid hydrophobic fat core containing antigen forms spontaneously. A typical composition of the lipid phase of the lipospheres consists of a neutral fat, such as tristearin or ethylstearate, and a phospholipid such as lecithin. A recombinant malaria antigen, R32NS1, containing epitopes derived from the circumsporozoite protein of *Plasmodium falciparum*, was incorporated in lipospheres together with lipid A as an adjuvant. After intramuscular injection of the formulation in rabbits, high levels of specific IgG antibodies to the antigen were still present 12 weeks after primary immunization. Several chemical and physical parameters that might be expected to influence the liposphere vaccine formulation were investigated. The particle size distribution of lipospheres depended on the fat to phospholipid (F/PL) molar ratio, and the immune response to liposphere-encapsulated R32NS1 was also dependent on the F/PL ratio. For F/PL ratios of ≤ 0.75 most of the lipospheres had an average diameter of less than 10 μm, while at F/PL ratios of ≥ 2.5 approximately 80% of the particles had an average size of 73 μm. Among the ratios tested, a maximal level of IgG antibody production was obtained at a F/PL ratio of 0.75, while at larger ratios decreased antibody production was observed. The effects of antigen dose and phospholipid composition were also examined.

INTRODUCTION

ADVANCES IN THE PRODUCTION of synthetic peptide and recombinant protein antigens has led to a plentiful supply of candidate antigens for human vaccines. Although these antigens are usually considered safe, they often have less immunogenicity than the original cell or organism from which they were derived and may

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require the use of an adjuvant to provide an effective vaccine. At the present time, the only adjuvants that are approved for use in humans by the U.S. Food and Drug Administration are "adsorbent" adjuvants consisting of aluminum compounds (often referred to as alum).[1-3] Aluminum based adjuvants are not uniformly effective and, although they have a reputation for safety, sterile abscesses and persistent nodules have been reported after their use, particularly in the event of inadvertent subcutaneous administration.[4]

Incomplete Freund's adjuvant (IFA), a water-in-oil (w/o) emulsion formulated with light mineral oil and a heterogeneous stabilizer containing an ester of mannitol and oleic acid (Arlacel A), has been proposed as an oil-based adjuvant for human use and has been administered to more than 30,000 individuals in the United States as an adjuvant for influenza, polio, and other viral vaccines.[5-7] Although IFA appears to be safe in populations of humans that have been available for long-term study,[8] the mineral oil of IFA is nonbiodegradable and the adjuvant can cause nodules or sterile abscesses in some subjects.[9-10] Arlacel A can be labile in the presence of certain antigens, resulting in release of free fatty acids and promotion of reactogenicity.[9] The discovery that Arlacel A causes tumors in male Swiss mice[10,11] has highlighted a potential for carcinogenicity of Arlacel A even though an increased incidence of tumors has not been observed in humans who have received IFA.[8] A variety of safety issues has caused considerable reduction in the use of IFA in human vaccines.[4]

Although liposomes and other oil-in-water (o/w) adjuvant formulations have been reported to have only "limited adjuvant activity" when compared to w/o preparations,[12] a successful human trial of alum-adsorbed liposomes containing monophosphoryl lipid A recently demonstrated that a formulation consisting of a combination of o/w and adsorbent adjuvants can have considerable safety and efficacy and may be useful in the development of a potential vaccine against the human malaria parasite (*Plasmodium falciparum*).[13]

Lipospheres represent a new type of lipid-based o/w encapsulation technology that may have potential usefulness in the formulation of human and veterinary vaccines. Lipospheres consist of water-dispersible microparticles composed of a solid hydrophobic fat core stabilized by one or more layers of phospholipids embedded in the particle surface. The objective of the present work was to determine the effects of different fats and phospholipids on the physical characteristics of potential liposphere formulations, and to evaluate the influence of a variety of different liposphere compositions on the immunogenicity of a recombinant protein antigen containing epitopes from the circumsporozoite protein of *P. falciparum*.

MATERIALS AND METHODS

Chemicals

Tristearin (Dynasan 118 microcrystalline triglyceride) was obtained from Hüls Troisdorf AG (Germany). Ethyl stearate was purchased from Sigma Chemical Co. (St. Louis, MO). Egg phosphatidylcholine (Coatsome NC-10S) was obtained from Nippon Oil & Fats Co. Ltd. (Japan), and dimyristoyl phosphatidylglycerol from Avanti Polar Lipids, Inc. (Alabaster, AL). Lipid A (isolated from *Salmonella minnesota* R595) was from List Biological Laboratories (Campbell, CA). Alum (Aluminum hydroxide, Rehsorptar Adsorptive Gel) was obtained from Armour Pharmaceutical Co. (Kankakee, IL).

Antigens

The two malaria antigens used in this study, R32NS1 and R32LR, were supplied under a Cooperative Research and Development Agreement by SmithKline Beecham Pharmaceuticals (King of Prussia, PA). R32SN1 is a fusion protein with the following amino acid sequence: [MDP$(NANP)_{15}$NVDP-$(NANP)_{15}$NVDP]$NS1_{81}$. The R32 refers to the 32 repeats of the tetrapeptide NANP interspersed with two tetrapeptide NVDP repeats from the immunodominant repeat region of the circumsporozoite (CS) protein of *P. falciparum*, and $NS1_{81}$ refers to 81 amino acids from the nonstructural protein of influenza virus. $NS1_{81}$ is added because it is thought to include human T-helper cell epitopes and to function as a carrier protein.[14] In the case of R32LR, R32 is linked to the first two amino acids, leucine and arginine (LR), from a nonsense reading

of the tetracycline gene of the vector.[15] The R32LR was used as capture antigen in the ELISA assay since it contains the same repeating units as the R32NS1 antigen used for immunization.[16]

Preparation of lipospheres

The lipid components (neutral fat, phospholipid, and lipid A) at the indicated molar ratios were dissolved in chloroform in a round-bottomed flask. Lipid A was included in the lipospheres because it has been used effectively by many laboratories to enhance humoral immunity to a wide range of antigens due to its adjuvant properties.[17] It was added to the lipid mixture at a weight ratio of 1 mg of lipid A per 500 mg of neutral fat. The organic solvent was evaporated using a rotary evaporator and the flask was placed in a desiccator under reduced pressure for 1 h to remove traces of residual solvent. The dry lipid mixture was then heated to 40°C to melt the ethyl stearate (m.p., 35°C). Warm phosphate-buffered saline (Dulbecco's PBS, GIBCO, NY) containing the R32NS1 malaria antigen (3.3 mg/ml) was added to give fat and phospholipid concentrations of 50 and 15 mg/ml, respectively, and the formulation was vigorously mixed for 1 min using a multiwrist shaker (Lab-Line Instruments Inc., Melrose Park, IL) until a homogeneous dispersion was obtained. The uniform milky-appearing suspension was immediately cooled below 20°C by immersing the flask in a dry ice-acetone bath for several seconds with continued shaking. Unencapsulated antigen was removed by centrifugation at 12,000 × *g* for 30 min at 20°C and washings with fresh PBS solution. Antigen encapsulation was ~80% as determined by a modified Lowry method for protein determination.[18] The antigen-free tristearin lipospheres shown in Fig. 1 were prepared in the same way except for the melting step, which was performed at 65°C (tristearin m.p. is approximately 60°C), and hot buffer solution (60–70°C), which was used in this case to disperse the melted fat vehicle and lipid mixture.

Particle size determination

Analysis of particle size distribution of lipospheres was performed using an LS 100 Coulter Counter Particle Size Analyzer (Coulter Corp., Hialeah, FL). This instrument, which can measure particles from 0.4 to 800 μm by particle size-dependent light diffraction patterns, is equipped with four main units: optical module, fluid module, computer, and printer. The optical module contains the laser light source, spatial filter and projection lens, diffraction sample cell, photodiode detectors, and Fourier lenses. The laser's radiation (750 nm wavelength, 4 mW operating power) passes through the spatial filter and projection lens to form a beam of collimated light. The diffraction sample cell shapes the suspension fluid and sample into a thin (3-mm) sheet which flows at right angles to the laser beam that passes through it. The Fourier lenses collect the diffracted light and focuse it onto three sets of detectors for low-, mid-, and high-angle scattering, forming an image of the entire diffraction pattern for each particle. The individual diffraction patterns from the many moving particles in the sample cell are then superimposed, creating a single integrated composite diffraction pattern that reflects the contribution from each particle and allows the determination of particle size distribution.

The computer (IBM compatible) uses an LS 130 software program (an integrated set of Microsoft window-based application programs) which controls the LS series instruments, processes the data, analyzes test results, and prints test reports. In the analysis mode, the particle size distribution plots can be computed as either number-, surface-, or volume-weighted distributions of particle diameter.

Immunization procedures

Rabbits (4 animals per group) were immunized intramuscularly at 0 and 4 weeks with 0.5–1.0 ml of liposphere formulations. The lipid A dose in all cases was 50 μg/rabbit. Each formulation was adsorbed on alum (0.8 mg/ml final concentration) prior to injection. The animals were bled before the primary immunization and every two weeks thereafter. The sera were collected and stored at −20°C until tested for antibody production.

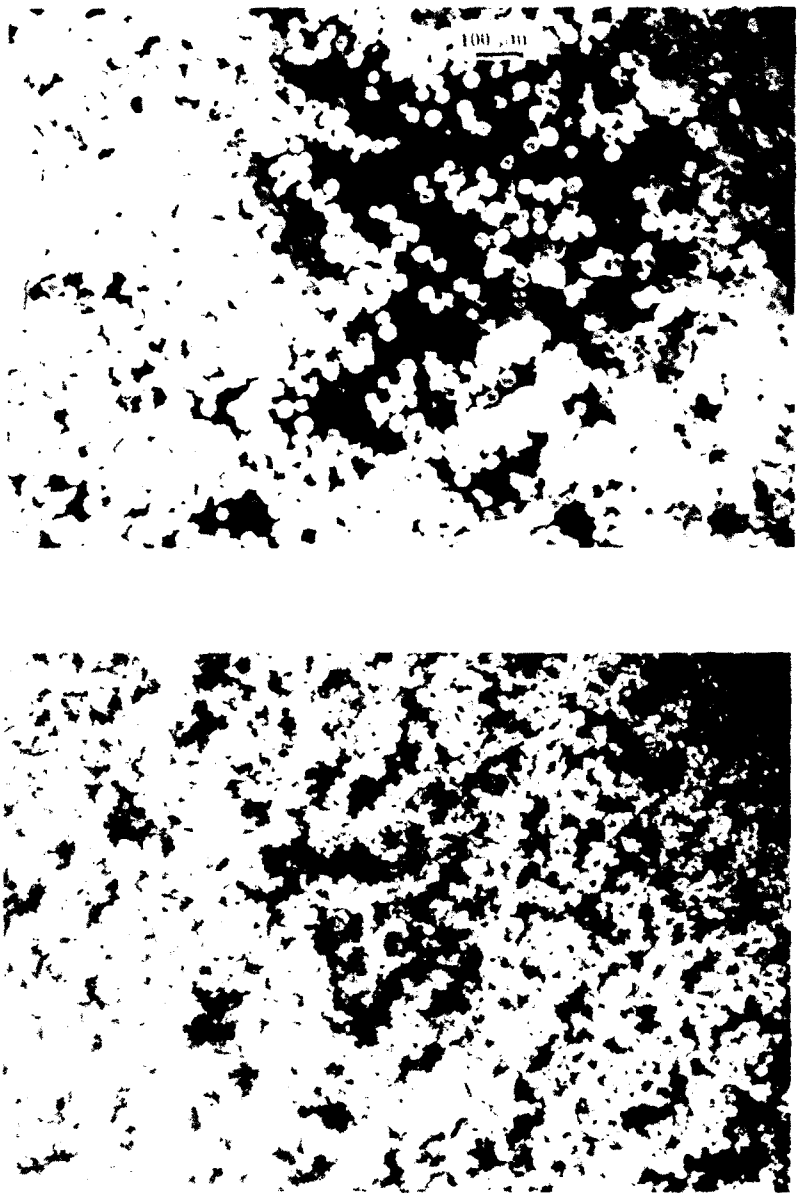

FIG. 1. Light microscopy of plain lipospheres composed of 4% tristearin (wt/vol) and 2% egg phosphatidylcholine (wt/vol) in phosphate-buffered saline.

All of the liposphere formulations injected containing lipid A (50 μg/rabbit) were nonpyrogenic and nontoxic, and they were generally well tolerated. No local inflammation or other side effects were observed at the site of administration during the 3-month period of the experiment.

Enzyme-linked immunosorbent assay (ELISA)

Solid phase ELISAs were carried out to evaluate the levels of IgG antibody activity against a capture antigen (R32LR) containing the same repeating units as the antigen that was used for immunization.[16] Assays were performed at room temperature in 96-well "U"-bottomed Immulon-2 polystyrene microplates (Dynatech

Laboratories, Alexandria, VA). The wells were coated with 0.1 μg of R32LR antigen dissolved in PBS. Approximately 18 h later, the contents of the wells were aspirated, filled with blocking buffer (0.5% casein, 0.01% thimerosal, 0.005% phenol red, and 1% Tween-20 in PBS), and held for 1 h at room temperature. Rabbit sera to be tested were diluted in 0.5% blocking buffer containing 0.025% Tween 20, and aliquots of each dilution were added to triplicate wells. After a 2-h incubation at room temperature, the contents of the wells were aspirated, washed three times with PBS-Tween 20 (0.05%), and 50 μg of horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Richmond, CA, diluted 1 : 1,000 in 0.5% blocking buffer containing 0.025% Tween 20) was added to each well. After 1 h the contents of the wells were aspirated, the wells were washed three times with PBS-Tween 20 washing solution, and 100 μl of ABTS-peroxidase substrate (Kirkgaard and Perry Laboratories, Inc., Gaithersburg, MD) was then added to each well. Absorbance was read at 405 nm 1 h after addition of peroxidase substrate using an automatic ELISA plate reader (Skatron, Norway).

RESULTS

Physical characteristics of lipospheres

A typical preparation of lipospheres composed of tristearin and lecithin contains particles that have a uniform spherical shape (Fig. 1). The particle size distribution of lipospheres depends on the neutral fat to phospholipid molar ratio (Table 1 and Fig. 2). Two populations of liposphere particles typically coexist, one in the size range of 1–10-μm diameter (population A), and a second population with a diameter between 10 and 80 μm (population B). Under conditions where the fat/PL ratio is high (≥2.5), the large particle population is predominant, whereas for fat/PL ratios of ≤ 0.75 most of the lipospheres have a diameter of less than 10 μm.

In the analysis display mode of the particle size analyzer used in this study (see Materials and Methods), the particle size distribution plots can be computed as either a number-, surface-, or volume-weighted distribution of particle diameter. The data shown in Fig. 2 represent *volume-weighted distributions plotted as a function* of particle diameter. The ordinate represents the number of particles multiplied by their volume given in arbitrary units and normalized to 100. The abscissa represents particle diameter and is divided into a fixed number of discrete bins, or slices, in a distribution pattern. The initial measurements that emerge from the instrument represent a light-scattering intensity-weighted diameter distribution. The number- and volume-weighted distributions are then obtained mathematically from the intensity-weighted results by applying the rules of light scattering. The autocorrelation function of scattered light intensity is proportional to the number of particles N_i having a diameter d_i, multiplied by the individual square volume of the particle $(V_i)^2$, which is proportional to $(d_i)^3$. That is, volume-weighted particle size distribution is obtained from the intensity-weighted distribution by dividing by V_i. To obtain the number-weighted distribution, one additional division of the intensity-weighted distribution is performed to give just N_i. Thus, the volume-weighted distribution

TABLE 1. PARTICLE SIZE DISTRIBUTION OF R32NS1-LIPOSPHERES AS A FUNCTION OF THEIR NEUTRAL FAT/PHOSPHOLIPID RATIO

	Population A		*Population B*	
Fat/PL molar ratio	*Volume % of particles*	*Average size (μm)*	*Volume % of particles*	*Average size (μm)*
0.75	70	6.2	30	44
1.25	45	6.3	55	61
2.50	23	8.5	77	73

The data represent the mean values of at least three batches of lipospheres as determined by the LS 100 Coulter Particle Size Analyzer (see Materials and Methods for more details).

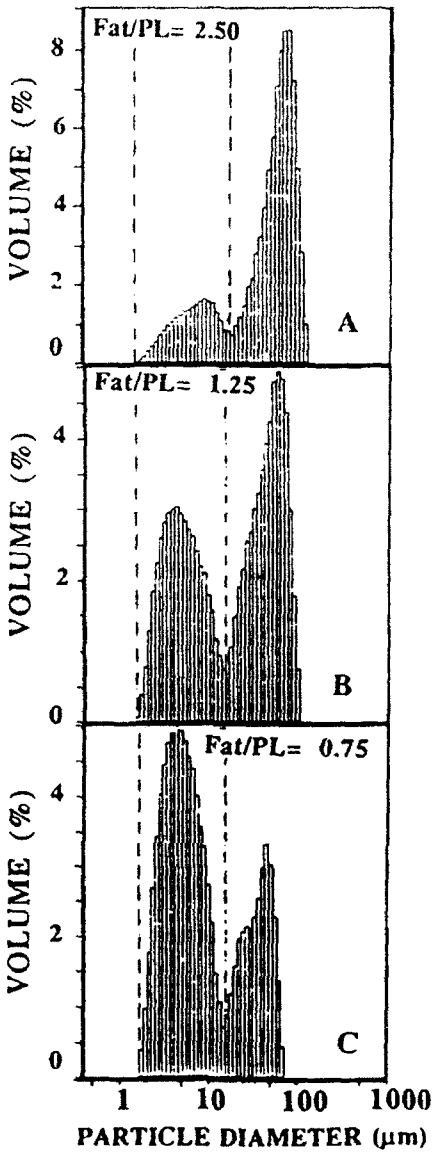

FIG. 2. Particle size distribution of lipospheres as function of their fat phospholipid molar ratio. Lipospheres containing the R32NS1 malaria antigen were made from ethylstearate and egg lecithin at different molar ratios (A = 2.50; B = 1.25; C = 0.75) and suspended in PBS. They were analyzed for particle size distribution using an LS 100 Coulter Particle Size Analyzer.

(weighted by diameter cubed), which takes into consideration the actual mass of the particles, is a more accurate approach to determine the total particle size distribution of a given sample.

For lipospheres, the average size of the particles increases with increasing fat/PL ratio (Table 1). For example, at a fat/PL ratio of 2.5, only 23% of the particles had an average size of 8.5 μm and the remaining 77% showed an average diameter of 73 μm (Fig. 2A). At lower fat/PL ratios, the volume percentage of large particles (population B, Table 1; Figs. 2B,C) decreases gradually, while the volume percentage of the smaller lipospheres (population A, Table 1) increases.

When an alternative adjuvant, alum (aluminum hydroxide), was examined by itself for comparative purposes under the same conditions, an average particle size of 14.4 ± 7.9 μm was found, with only 2% of the alum particles being larger than 33.5 μm in diameter (data not shown).

Effect of antigen dose on immune response to encapsulated antigen

The role of antigen dose in the immune response to liposphere-encapsulated malaria antigen, LS(R32NS1), is shown in Figs. 3 and 4. IgG antibodies against R32NS1 were observed to occur in rabbits 2 weeks after the primary immunization, and titers were enhanced by a boosting injection of encapsulated antigen given at 4 weeks after the primary injection. A five-fold increase in the antibody titer was obtained in rabbits receiving two 10-μg doses of LS(R32NS1) compared to those injected with two 1-μg doses (Fig. 3). Two doses of 100 μg of antigen did not result in further increase in the IgG antibody level above that obtained with two 10-μg doses, and these two doses were not significantly different in their antibody activity ($p = 0.284$). Antibody titers in rabbits receiving the two 10-μg and two 100-μg doses were detected even at 1:1,600 serum dilution (Fig. 4).

It is worth noting that the IgG antibody ELISA titers obtained on immunizing rabbits with LS(R32NS1) were superior to those obtained following similar immunizations with the free antigen absorbed to alum, which showed no antibody activity at the same antigen concentrations (Richards and Alving, unpublished results).

Effect of phospholipid composition on immunogenicity

The time course of IgG antibody activity as a function of liposphere phospholipid composition is shown in Fig. 5. Incorporation of a negatively charged phospholipid, dimyristoyl phosphatidylglycerol (DMPG), in the

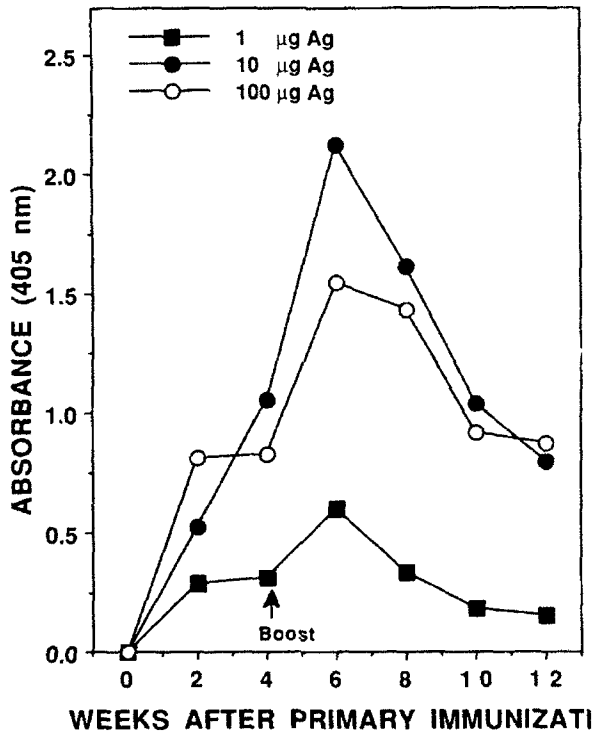

FIG. 3. Effect of antigen dose on IgG antibody activity in rabbits immunized with R32NS1 malaria antigen encapsulated in lipospheres. Each point represents the mean ELISA absorbance value (4 rabbits per group) after subtraction of the preimmune value at a serum dilution of 1 : 200. Each rabbit was immunized at 0 and 4 weeks with the indicated doses of antigen. Fat/phospholipid molar ratio = 2.50. The values in the curves for 10 μg and 100 μg were not significantly different ($p = 0.28$; two-tailed Student's t-test).

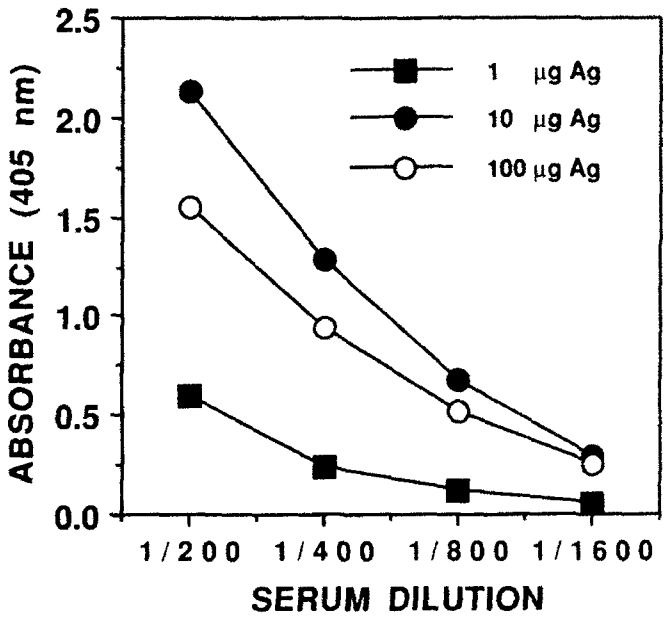

FIG. 4. ELISA IgG antibody activity at 6 weeks in rabbits immunized twice (at 0 and 4 weeks) with 1-, 10-, and 100-µg doses of R32NS1 malaria antigen encapsulated in lipospheres. Each point represents the mean ELISA antibody response (4 rabbits per group) at the indicated serum dilution after subtraction of the preimmune value. Fat phospholipid molar ratio = 2.50. The values in the curves for 10 µg and 100 µg were not significantly different ($p = 0.28$; two-tailed Student's *t*-test).

liposphere lipid phase caused a significant increase in the antibody response to the encapsulated R32NS1 antigen (Figs. 5 and 6). This effect of DMPG was especially pronounced at 6 weeks, 2 weeks after the boosting injection.

Effect of fat/phospholipid molar ratio on immunogenicity

The influence of fat/PL molar ratio on the immune response to liposphere-encapsulated R32NS1 is shown in Figs. 7 and 8. Among the fat/PL molar ratios tested (0.75, 1.25, and 2.50), the maximal level of IgG antibody activity was obtained at a ratio of 0.75 (Fig. 7). At larger values of fat/PL a decrease in antibody level was observed. Except for one value at week 8, no significant differences in IgG antibodies were observed at fat/PL ratios of 1.25 and 2.50.

DISCUSSION

The results presented in this study demonstrate that excellent immunogenic efficacy can be achieved by using liposphere-based formulations and that lipospheres represent a novel vaccine carrier. Lipospheres can be viewed as an o/w system that has intermediate characteristics between liposomes and oil emulsions. Lipospheres differ from liposomes in that their internal phase is a hydrophobic fat core, compared to an internal aqueous phase in liposomes. The fat that is present in lipospheres differs from the oil widely employed in emulsions, such as IFA or intravenous fat emulsion, in that the fat of lipospheres is solid at the time of manufacture at room temperature and may even be solid at 37°C. In contrast to certain oil emulsions (including IFA), the liposphere approach utilizes only naturally occurring biodegradable lipid constituents (*e.g.*, triglycerides and lecithin). The surface activity of lipospheres is provided by the phospholipid component embedded in the particle surface.

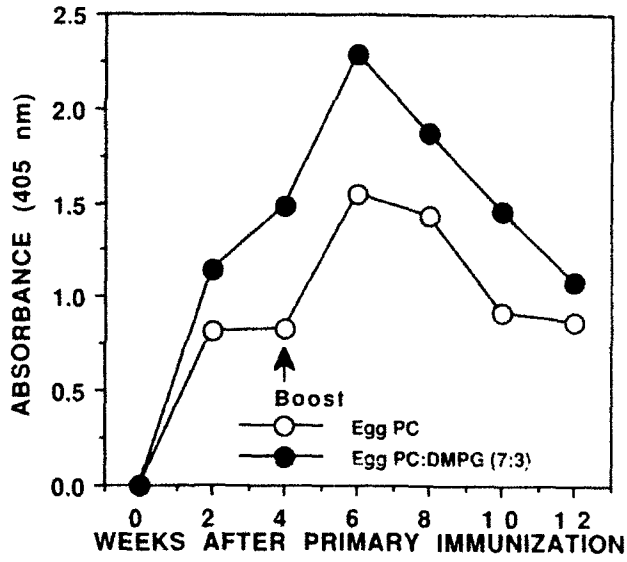

FIG. 5. Effect of phospholipid composition on immunogenicity of lipospheres containing encapsulated R32NS1. Two formulations made of ethylstearate and differing in their phospholipid composition (PC compared to PC:DMPG, 7 : 3 molar ratio) were injected in rabbits (4 per group) at 0 and 4 weeks, and the IgG antibody responses in their sera were measured with time. Each point represents the mean ELISA absorbance value after subtraction of the preimmune value at a serum dilution of 1 : 200. Antigen dose = 100 μg/rabbit. Fat phospholipid molar ratio = 2.50. All the values in both curves were significantly different ($p < 0.05$; two-tailed Student's t test).

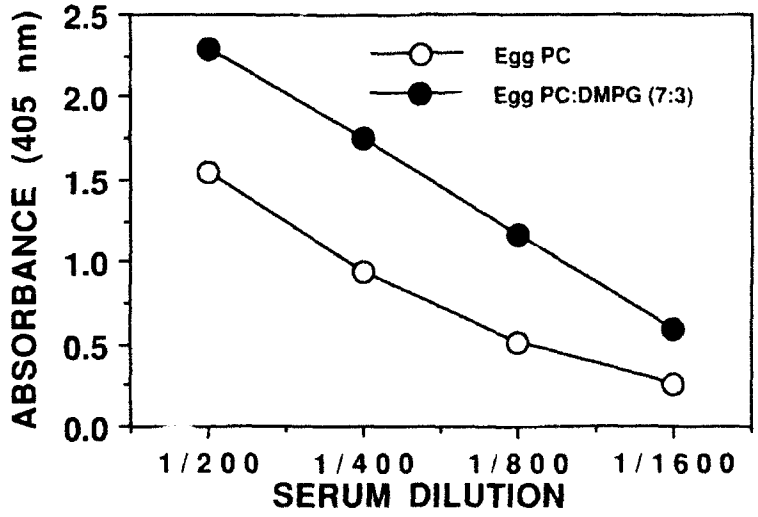

FIG. 6. IgG antibody activity at 6 weeks in rabbits immunized twice (at 0 and 4 weeks) with 100-μg doses of R32NS1 malaria antigen encapsulated in lipospheres composed of ethylstearate and PC or PC:DMPG (7 : 3) at a fat phospholipid molar ratio of 2.50. Each point represents the mean antibody response (4 rabbits per group) at the indicated serum dilution after subtraction of the preimmune value. All the values in both curves were significantly different ($p < 0.05$; two-tailed Student's t test).

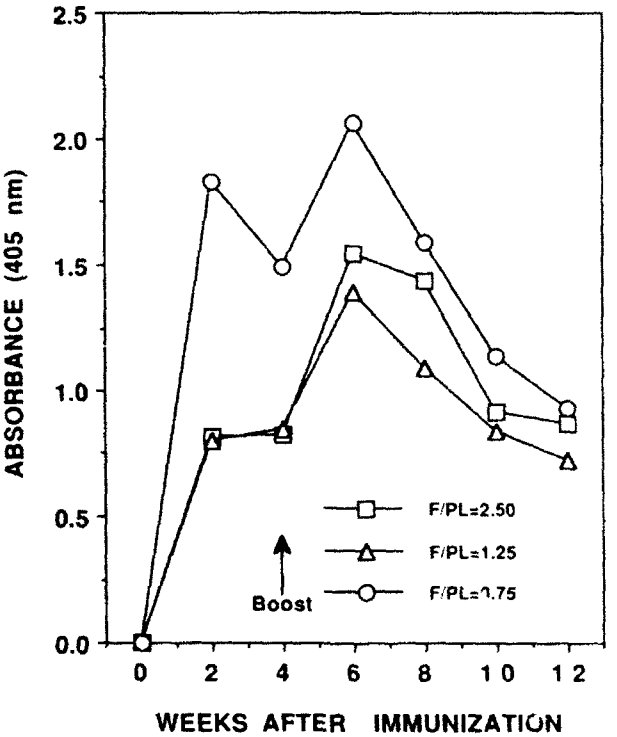

FIG. 7. Effect of fat/phospholipid molar ratio on the immunogenicity of lipospheres containing encapsulated R32NS1 in rabbits injected with two 100-μg antigen doses at 0 and 4 weeks. Each point represents the mean absorbance value after subtraction of the preimmune value at a serum dilution of 1 : 200. Except for one point at week 8, the values in the curves for F/PL = 1.25 and 2.50 were not significantly different ($p = 0.803$).

It was previously shown that a recombinant antigen (R32NS1) containing tetrapeptide epitopes derived from the circumsporozoite protein of *P. falciparum* was poorly immunogenic in humans when injected alone as an aqueous solution, or when adsorbed on alum.[14] The immunogenicity of R32NS1 was markedly increased in humans when the antigen was encapsulated in alum-adsorbed liposomes containing monophosphoryl lipid A.[13] The present study demonstrates that very high levels of IgG antibody production, comparable to those observed with liposomes, appeared in the serum of rabbits immunized with lipospheres containing R32NS1 and lipid A, especially after the boosting injection. The major purpose of this study was to characterize the properties of the lipospheres by investigating the influence of the physical structure of lipospheres on the immunogenicity of R32NS1.

An interesting correlation was observed between the liposphere fat/PL molar ratio, particle size, and immunogenicity. Low fat/PL ratios (≤0.75), which result in the formation of lipospheres of small particle size (70% with an average size of ~6 μm), resulted in increased antibody titers (Fig. 7). Although the reason for this phenomenon is unknown, a possible explanation may be the occurrence of better antigen orientation and epitope exposures in small lipospheres when compared to large lipospheres because of higher surface curvature. A similar phenomenon has also been reported with small liposomes which generated higher antibody titers against encapsulated antigen than large liposomes.[19]

The immunogenicity of liposphere-associated R32NS1 was increased by the incorporation of a negatively charged lipid (DMPG) in the liposphere lipid phase. Effects of lipid charge have also been observed with certain antigens in liposomes. Negatively charged liposomes produced a better immune response to diphtheria toxoid than positively charged liposomes.[20] However, when liposomes were prepared with other antigens,

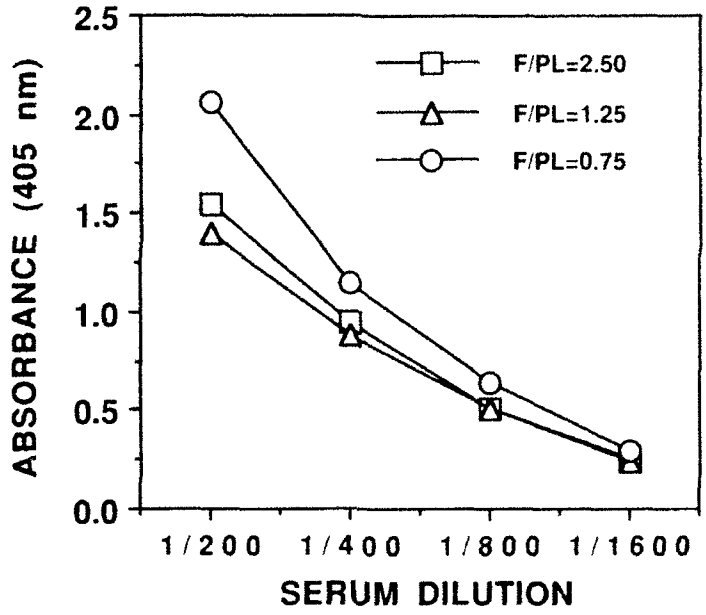

FIG. 8. IgG antibody activity at 6 weeks in rabbits immunized twice (at 0 and 4 weeks) with 100-μg doses of liposphere-encapsulated R32NS1 having fat-to-phospholipid molar ratios of 0.75, 1.25, and 2.50. Each point represents the mean antibody response (4 rabbits per group) at the indicated serum dilution after subtraction of the preimmune value. Except for one point at week 8, the values in the curves for F/PL = 1.25 and 2.50 were not significantly different ($p = 0.803$).

positively charged liposomes worked equally as well as those bearing a negative charge.[21,22] Further studies will determine whether negative charges in lipospheres have general abilities to enhance immunogenicity or whether, as with liposomes, charge effects are dependent on individual antigen composition.

Binding of antigen to a surface, or presentation of a special type of surface for antigen adsorption, appears to be a property that is found with many agents that are reported to have adjuvant activities.[12] The data obtained with the liposphere-encapsulated antigen in the present study suggests that a relationship exists between the physicochemical surface properties of lipospheres and their ability to serve as adjuvants. It has been proposed by Hunter *et al.*[23] that the abilities of surfactants to act as adjuvants are dependent on their capability of concentrating adjuvant and immunogen on hydrophobic surfaces, where they are more effectively presented to cells of the immune system. Although this suggests that surfactants can have adjuvant properties, it is cautioned that selectivity must be exercised in devising safe and efficacious adjuvant systems because of the existence of thousands of surface-active agents. As pointed out elsewhere, "To immunologists not familiar with surface science, choosing adjuvant-active substances without guidelines is nearly impossible."[12]

The present study suggests that lipospheres are a unique formulation that may have useful applications as carriers of vaccines. Among the advantages of lipospheres are the following. First, after formulation the lipospheres can be used in a "dry" form without concern about release of antigen or adjuvant such as might occur with a wet vesicle suspension such as liposomes. Second, there is no need for the presence of a stabilizer such as Arlacel A that is utilized in Freund's adjuvant. Third, the manufacturing techniques for lipospheres are relatively simple, thereby leading to the expectation that scaling-up procedures might be easily accomplished. Fourth, the ingredients used for manufacture of lipospheres are relatively inexpensive. Fifth, if a lipophilic adjuvant, such as lipid A, should prove to be necessary with a given antigen, it can also be incorporated into the fat phase of the lipospheres.

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